Characterization of the Signal Transduction Pathway Activated in Human Monocytes and Dendritic Cells by MPIF-1, a Specific Ligand for CC Chemokine Receptor 1

Bernardetta Nardelli, H. Lee Tiffany, Gary W. Bong, Pamela A. Yourey, Diana K. Morahan, Yuling Li, Philip M. Murphy and Ralph F. Alderson

*J Immunol* 1999; 162:435-444; ;
http://www.jimmunol.org/content/162/1/435

**References** This article cites 36 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/162/1/435.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Characterization of the Signal Transduction Pathway Activated in Human Monocytes and Dendritic Cells by MPIF-1, a Specific Ligand for CC Chemokine Receptor 1

Bernardetta Nardelli,1* H. Lee Tiffany,§ Gary W. Bong,* Pamela A. Yourey,† Diana K. Morahan,* Yuling Li, Philip M. Murphy,§ and Ralph F. Alderson†

The receptor specificity and signal transduction pathway has been identified and characterized for a truncated form of myeloid progenitor inhibitory factor-1 (MPIF-124-99). MPIF-1 binds specifically to sites, in particular CCR1, shared with macrophage inflammatory protein-1α (MIP-1α) on the surface of human monocytes and dendritic cells, as inferred by its ability to compete for [125I]MIP-1α, but not for [125I]MIP-1β or [125I]monocyte chemotactic protein-1(MCP-1) binding to intact cells. Based on calcium flux, MPIF-1 is an agonist on CCR1-transfected HEK-293 cells, monocytes, and dendritic cells, but not on CCR5-, CCR8-, or CX3CR1-transfected cells. The inhibitory effect of guanosine 5′-O-(3-thio-triphosphate) (GTP-γS) or pertussis toxin pretreatment on MPIF-1 binding and calcium mobilization, respectively, indicates the involvement of G proteins in the interaction of MPIF-1 and its receptor(s). The increase in intracellular free calcium concentration following MPIF-1 treatment is mainly due to the influx of calcium from an extracellular pool. However, a portion of the intracellular free calcium concentration is derived from a phospholipase C inhibitor-sensitive intracellular pool. MPIF-1 induces a rapid dose-dependent release of [3H]arachidonic acid from monocytes that is dependent on extracellular calcium and is blocked by phospholipase A2 (PLA2) inhibitors. Furthermore, PLA2 activation is shown to be necessary for filamentous actin formation in monocytes. Thus, the MPIF-1 signal transduction pathway appears to include binding to CCR1; transduction by G proteins; effector function by phospholipase C, protein kinase C, calcium flux, and PLA2; and cytoskeletal remodeling. The Journal of Immunology, 1999, 162: 435–444.

Chemokines are structurally related proteins containing four conserved cysteines. Depending on the positions of the first two cysteines, the chemokines are grouped into two major families (1, 2), CXC chemokines, with the first two cysteines separated by a nonconserved amino acid, are mainly chemoattractant for granulocytes and T lymphocytes. CC chemokines, in which the first two cysteines are adjacent, are biologically active on a variety of cell types.

All the known chemokines signal through two groups of seven transmembrane receptors, CXCR and CCR, belonging to the rhodopsin-G protein-coupled receptor superfamily (3). The extracellular domain event results in the dissociation of the α and βy subunits of the G protein, initiating the activation of several different signal transduction pathways, such as adenylate cyclase, protein kinase C (PKC),2 tyrosine and mitogen-activated protein kinases, and phospholipases A2, C, and D (PLA2, PLC, and PLD, respectively) (3, 4). Following activation, chemokine receptors rapidly become unresponsive to further stimulation. This desensitization process is thought to be caused by phosphorylation on residues present in the carboxyl tail of the protein and receptor internalization (5, 6).

Monocytes and dendritic cells, which play an important role in the pathogenesis of inflammation and in Ag presentation, are responsive to chemokines. Monocytes express the chemokine receptors CCR1, CCR2, CCR5, CCR8, CXCR2, and CXCR4 (7). Of the CC chemokines, MCP-1 is a potent monocyte activator in vitro in that following binding to CCR2, it induces intracellular calcium flux, actin polymerization, oxidative burst, lysosomal enzyme, and cytokine release (8, 9). In comparison, MIP-1α, which binds CCR1 and CCR5, and MIP-1β, which binds CCR5, are weaker inducers of exocytosis and do not stimulate oxidative burst and cytokine release (10, 11), suggesting perhaps functional differences between the receptors (12).

The expression of chemokine receptors in dendritic cells depends on the developmental pathways and the stage of differentiation of the cells. Monocyte-derived dendritic cells express CCR1, CCR2, CCR5, CXCR1, CXCR2, and CXCR4 (7), while CD34 derived dendritic cells also express CCR6 (13). CCR2 receptors in dendritic cells appear to have a substantially reduced functional activity, since calcium flux and chemotaxis induced by MCP-1 in this cell type are minimal (7). The effect of chemokines on dendritic cells seems to be restricted to chemotactic activity (7). Thus, chemokines might participate in the trafficking of dendritic cells, such as homing of progenitor cells to the periphery and migration of mature cells to lymphoid tissues, and in the recruitment of dendritic cells to inflamed mucosal sites (14, 15).

Myeloid progenitor inhibitor factor 1 (MPIF-1), a 99-amino acid CC chemokine containing six cysteines, was recently identified in a cDNA library derived from human aortic endothelium cells (16).
The cysteines at positions 33, 34, 57, and 73 are common to the other CC chemokines, while those at positions 44 and 84 are not conserved. MPIF-1 is most homologous to the MIP chemokine subgroup, with identities of 51 and 41% to MIP-1α and MIP-1β, respectively, while having <40% identity to the MCP subgroup. Although MPIF-1 has been characterized to have chemotactic activity on monocytes and dendritic cells (16, 17), (B. Nardelli, unpublished observations) and to inhibit colony formation of bone marrow-derived low proliferative potential colony-forming cells (16), little is known about its receptor specificity and mechanism of action.

The aim of the present report is to investigate the receptor specificity and signal transduction pathway of a novel amino-terminal truncated form of MPIF-1 (amino acids 24–99), herein designated MPIF-1, in monocytes and dendritic cells. In addition, we compared the receptor specificity and the biological activities of MPIF-1 to the full-length MPIF-1,99 in monocytes. Using competitive binding of iodinated chemokines and receptor desensitization, we demonstrate that MPIF-1 binds and activates CCR1. Furthermore, we demonstrate that monocytes respond to MPIF-1 treatment by the activation of PLA2, a rapid increase in filamentous actin (F-actin) formation, and the release of [3H]arachidonic acid ([3H]AA).

Materials and Methods

Materials

Granulocyte-macrophage CSF, IL-4, MIP-1a, MIP-1β, MCP-1, and MCP-3 were purchased from PeproTech (Rocky Hill, NJ), and 130-2, RANTES, and full-length MPIF-1 were obtained from R&D Systems (Minneapolis, MN). Iodinated MIP-1α, MIP-1β, and MCP-1 (2000 Ci/ mmol) were purchased from Amersham (Arlington Heights, IL). MPIF-1 was iodinated with [125I]iodine, hydrogen peroxide, and lactoperoxidase (2200 Ci/mmoll, Amersham). Pertussis toxin, NDGA, U73122, U73343, and staurosporine were purchased from Calbiochem (La Jolla, CA). PMA and 4-BBP were purchased from Sigma (St. Louis, MO). Manoolide was purchased from RBI (Natick, MA).

MPIF-1 expression and purification

The coding sequence of MPIF-1 was amplified from a human aortic endothelial library by PCR, during which unique restriction sites (SpI/His- dIII) were introduced, thereby allowing the gene to be inserted into the expression vector pQE7. The resulting plasmid DNA was used to transform Escherichia coli M15 Rep4 host cells. The bacterial transformants were grown in Luria Bertoni medium containing ampicillin and kanamycin. Induction was performed at 1 mM isopropyl β-[Iacetyl]-thiogalactoside for 3 h. For large scale production, a semi-defined medium without antibiotics was used. MPIF-1 was produced as an insoluble protein deposited within inclusion bodies. MPIF-1 appears as a 9-kDa protein on reduced SDS-PAGE. For large scale production, a semi-defined medium without antibiotics was used. MPIF-1 was produced as an insoluble protein deposited within inclusion bodies. MPIF-1 appears as a 9-kDa protein on reduced SDS-PAGE. The recovery method for MPIF-1 used a series of procedures to separate the host contaminants through cell lysis, washes, and differential centrifugations to isolate inclusion bodies that contain partially purified MPIF-1. E. coli cell paste was suspended in a buffer containing 100 mM Tris (pH 7.4) and 25 mM EDTA. The cells were lysed by passing twice through a microfluidizer (Microfluidics, Newton, MA) at 6000–8000 psi. The lysed sample was mixed with NaCl to a final concentration of 0.5 M and then centrifuged at 7000 × g for 15 min. The resulting pellet was washed again with the same buffer plus 0.5 M NaCl and then centrifuged at 7000 × g for 15 min.

The partially purified inclusion bodies were then resuspended for 2–4 h at 20–25°C in 1.75 M guanidine hydrochloride containing 100 mM Tris (pH 7.4) and 25 mM EDTA. The sample was placed at 2–8°C overnight and subsequently centrifuged at 30,000 × g at the next day. The supernatant (1.75 M guanidine hydrochloride extract) was mixed vigorously for 30 min at 4°C with 10 vol of a buffer containing 50 mM sodium acetate (pH 4.5), 125 mM sodium chloride, and 2 mM EDTA. Afterward the mixture was placed at 4°C without mixing for 1–48 h before the chromatographic purification steps described below.

The diluted MPIF-1 sample was clarified using a 0.22-μm pore size sterile filter (Pall Ultrafine Filtration, East Hills, NY). The MPIF-1 protein was then chromatographed over a strong cation exchange (POROS HS-50) column. The HS column was washed first with 6 column vol of a buffer containing 50 mM sodium acetate (pH 6.0) and 300 mM sodium chloride. The bound protein was eluted using 3–5 column vol of a stepwise gradient of 500, 750, 1000, and 1500 mM sodium chloride in 50 mM sodium acetate at pH 6.0.

The HS fraction eluted with 0.5 M sodium chloride was diluted 2-fold with water. The diluted sample was applied to a set of strong anion (POROS HQ-50) and weak cation (POROS CM-20) exchange columns in tandem mode. Both columns were washed with 50 mM sodium acetate (pH 6.0) and 150 mM sodium chloride. The carboxymethyl column was eluted with a 10- to 20-column vol linear gradient of 150-1000 mM NaCl. Fractions containing purified MPIF-1, as analyzed through SDS-PAGE and reverse phase HPLC, were combined. The carboxymethyl-purified MPIF-1 was loaded onto a size exclusion (Sephacryl S-100 HR) column for final polishing.

Cells

PMBC were purified from single donor Leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes were isolated from PMBC by counterflow centrifugal elutria- tion. Dendritic cells were generated by culturing elutriated monocytes in RPMI containing 10% FBS supplemented with granulocyte-macrophage CSF (50 ng/ml) and II-4 (20 ng/ml) as previously described (18). Following 3–5 days in culture, the phenotype of the cells as identified by FACS analysis was >90% CD14+ >90% MHC class II, <10% CD14−, <5% CD3+, and <5% CD20+. HEK-293 cell lines expressing human CCR1 or human CCR5 and mouse pre-B cell lymphoma 4D4 cells expressing human CCR8 have been previously described (19–21). 4D4 pre-B cells were maintained in RPMI 1640 containing 10% FBS, 50 μM 2-ME, and 1 mg/ml G-418 at 37°C in 5% CO2 and 100% humidity. HEK-293 cell lines were maintained in DMEM with 10% FBS supplemented with 2 μg/ml G-418. THP-1 cells were obtained from American Type Culture Collection (Manassas, VA).

Whole cell binding assays

Competition binding studies were conducted using 1 × 106 monocytes, 5 × 104 dendritic cells, or 2 × 106 THP-1 cells/well. Cells were resuspended in binding buffer (Ham’s F-12 medium containing 0.5% BSA and 0.1% sodium azide) and transferred to a 96-well U-bottom plate (Costar, Cambridge, MA). The iodinated chemokine was then added in the absence or the presence of cold competitor chemokines in a total volume of 100 μl of binding buffer. The binding reaction was conducted for 90 min at 24°C with gentle agitation. Subsequently, the cells were pelleted by centrifugation at 1300 rpm for 5 min, and the supernatant was removed. The cells were washed with cold PBS (4°C) and then transferred to an opaque 96-well plate (Microfluor, Nunc, Newbury Park, CA). Following a further centrifugation, the cells were resuspended in 100 μl of liquid scintillant and then counted.

Membrane binding assay

CCR1, CCR2b, and CCR5 membranes were purchased from Receptor Bi- ology (Beltsville, MD), and the assays were conducted as suggested by the manufacturer. Briefly, CCR1-containing (25 μg), CCR2b-containing (8.2 μg), or CCR5-containing (50 μg) membranes were incubated in a 96-well plate with [125I]MIP-1α (50 μM), [125I]MCP-1 (55 μM), or [125I]MIP-1β (500 μM), respectively, in the presence or the absence of cold competitor chemokines in a total volume of 100 μl of binding buffer (50 mM HEPES (pH 7.2), 5 mM MgCl2, 1 mM CaCl2, 0.5% BSA, 0.002% sodium azide, and protease inhibitors). Following an incubation for 90 min at 24°C, the membranes were centrifuged at 2500 rpm for 10 min. The supernatant was decanted, 100 μl of binding buffer (4°C) containing 0.5 μM NaCl was added, and the membranes were transferred to an opaque plate. Following two additional rinses, the membranes were resuspended in 100 μl of scin- tillant and counted.

Measurement of changes in [Ca2+]i

Monocytes, dendritic cells, or THP-1 cells were resuspended in Ca2+ flux buffer (10 mM HEPES buffer (pH 7.4) containing 1 mM CaCl2, 2 mM MgSO4, and 5 mM glucose) and loaded with fura-2 (Molecular Probes, Eugene, OR; 0.2 nmol/106 cells) for 30 min at 37°C. The cells were then washed, resuspended in the labeling buffer, and treated with chemokine(s). Fura-2 fluorescence was measured on a fluorescence spectrophotometer (F-2000, Hitachi, San Jose, CA), and the ratio of the emission at 340 and 380 nm was determined. Calcium flux in cell lines transfected with CCR1, CCR5, and CCR8 was measured exactly as described previously (21).
FIGURE 1. Monocytes, dendritic cells, and THP-1 cells express shared binding sites for MIP-1α and MPIF-1. Competition of iodinated MIP-1α (A, D, and G), MIP-1β (B, E, and H), or MCP-1 (C, F, and I) binding to monocytes (A–C), dendritic cells (D–F), or THP-1 cells (G–I) by the corresponding unlabeled chemokine (open square) or MPIF-1 (closed square). Cells were incubated in 100 μl of binding buffer for 90 min at 24°C with 500 pM labeled chemokine and the unlabeled competitors at the indicated concentrations. The data points (n=4) represent the level of specific binding obtained at the indicated concentration of cold competitor, expressed as a percentage of the maximum bound iodinated chemokine.

Release of [3H]AA

The [3H]AA release assay was adapted from the protocol of Dumuis et al. (22). Freshly isolated monocytes were incubated overnight in RPMI containing 10% FBS and 1 μCi/ml of [3H]AA (205 Ci/mmol; Amersham). At the end of the incubation period, the cells were washed three times (10 min each) with RPMI containing 0.2% fatty acid-free BSA and then resuspended at the appropriate cell density in the same medium. Following the treatment period, the supernatant was removed, centrifuged, and counted. The remaining cell associated label was quantitated by solubilizing the cells in 0.2 M NaOH and counting the lysates.

Measurement of cAMP formation

The level of cAMP in the cultures of monocytes or dendritic cells was determined by ELISA according to the manufacturer’s instructions (Amersham).

Measurement of F-actin

Actin polymerization was analyzed as previously described (23). Monocytes (100-μl aliquots of 10⁷ cells/ml) were preincubated with appropriate chemicals in HBSS containing 0.05% BSA and then treated with the chemokines at 37°C. The reaction was stopped by addition of fixing-staining buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.0, containing 8% (w/v) paraformaldehyde, 0.2 mg/ml lysophosphatidylcholine, and 0.3 μM Oregon Green 488 phalloidin (Molecular Probes). The cells were stained for 30 min at room temperature, washed, resuspended in PBS, and analyzed by FACSScan (Becton Dickinson, San Jose, CA). The mean fluorescence intensity per cell was used as a measure of F-actin content per cell. Results are expressed as the relative fluorescence index, i.e., the ratio of the mean fluorescence intensity of chemokine-treated cells to the mean fluorescence intensity of control cells.

Results

MPIF-1 and MIP-1α bind to a shared site on monocytes and dendritic cells

In competition binding studies using monocytes, MIP-1α and MPIF-1 at a concentration of 40 or 1000 nM, respectively, displaced 50% of the bound [125I]MIP-1α (Fig. 1A), while in dendritic or THP-1 cells, 50% displacement was achieved with 1 nM MIP-1α or with between 200–400 nM MPIF-1 (Fig. 1, D and G). Concentrations of MIP-1α in the range of 10–100 nM produced maximal displacement (70–90%) of [125I]MIP-1α in all three cell types, while maximal displacement of [125I]MIP-1α was achieved with concentrations of MPIF-1 between 100-1000 nM. Although specific [125I]MIP-1β binding was observed in all three cell types, MPIF-1, even at concentrations as high as 1000 nM, did not displace a significant amount of the bound [125I]MIP-1β (Fig. 1, B, E, and H). The binding of [125I]MCP-1 on monocytes was displaced by 50 or 85% with 0.7 or 100 nM MCP-1, respectively (Fig. 1C). In contrast, in monocytes MPIF-1 did not displace bound [125I]MCP-1 at any concentration tested. In dendritic cells, 100 nM MPIF-1 displaced approximately 20% of the bound [125I]MCP-1, suggesting that the iodinated ligand was binding to a low affinity site (Fig. 1, F and I). In contrast, the displacement of [125I]MCP-1 on THP-1 cells was nearly fully achieved with 0.5 nM MPIF-1. However, the maximal level of displacement achieved with 1000 nM MPIF-1 was 50%, in contrast to 90% competition in the presence of 100 nM MCP-1. Based on the receptor specificity of MIP-1α, MIP-1β, and MCP-1, the results of the above cross-competition studies suggest that MPIF-1 binds to CCR1 on all three cell types and possibly to CCR2 on THP-1 cells.

MPIF-1 binds to CCR1, but not to CCR2b or CCR5

To further delineate the receptor specificity of MPIF-1, cross-competition studies were conducted on membranes from cell lines expressing only one type of chemokine receptor. Using membranes from HEK-293 cells expressing CCR1, the ability of MPIF-1 to compete with [125I]MIP-1α binding was investigated (Fig. 2A). The competition curves observed with increasing concentrations of

Measurement of F-actin

Actin polymerization was analyzed as previously described (23). Monocytes (100-μl aliquots of 10⁷ cells/ml) were preincubated with appropriate chemicals in HBSS containing 0.05% BSA and then treated with the chemokines at 37°C. The reaction was stopped by addition of fixing-staining buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.0, containing 8% (w/v) paraformaldehyde, 0.2 mg/ml lysophosphatidylcholine, and 0.3 μM Oregon Green 488 phalloidin (Molecular Probes). The cells were stained for 30 min at room temperature, washed, resuspended in PBS, and analyzed by FACSScan (Becton Dickinson, San Jose, CA). The mean fluorescence intensity per cell was used as a measure of F-actin content per cell. Results are expressed as the relative fluorescence index, i.e., the ratio of the mean fluorescence intensity of chemokine-treated cells to the mean fluorescence intensity of control cells.

Results

MPIF-1 and MIP-1α bind to a shared site on monocytes and dendritic cells

In competition binding studies using monocytes, MIP-1α and MPIF-1 at a concentration of 40 or 1000 nM, respectively, displaced 50% of the bound [125I]MIP-1α (Fig. 1A), while in dendritic or THP-1 cells, 50% displacement was achieved with 1 nM MIP-1α or with between 200–400 nM MPIF-1 (Fig. 1, D and G). Concentrations of MIP-1α in the range of 10–100 nM produced maximal displacement (70–90%) of [125I]MIP-1α in all three cell types, while maximal displacement of [125I]MIP-1α was achieved with concentrations of MPIF-1 between 100-1000 nM. Although specific [125I]MIP-1β binding was observed in all three cell types, MPIF-1, even at concentrations as high as 1000 nM, did not displace a significant amount of the bound [125I]MIP-1β (Fig. 1, B, E, and H). The binding of [125I]MCP-1 on monocytes was displaced by 50 or 85% with 0.7 or 100 nM MCP-1, respectively (Fig. 1C). In contrast, in monocytes MPIF-1 did not displace bound [125I]MCP-1 at any concentration tested. In dendritic cells, 100 nM MPIF-1 displaced approximately 20% of the bound [125I]MCP-1, suggesting that the iodinated ligand was binding to a low affinity site (Fig. 1, F and I). In contrast, the displacement of [125I]MCP-1 on THP-1 cells was nearly fully achieved with 0.5 nM MPIF-1. However, the maximal level of displacement achieved with 1000 nM MPIF-1 was 50%, in contrast to 90% competition in the presence of 100 nM MCP-1. Based on the receptor specificity of MIP-1α, MIP-1β, and MCP-1, the results of the above cross-competition studies suggest that MPIF-1 binds to CCR1 on all three cell types and possibly to CCR2 on THP-1 cells.

MPIF-1 binds to CCR1, but not to CCR2b or CCR5

To further delineate the receptor specificity of MPIF-1, cross-competition studies were conducted on membranes from cell lines expressing only one type of chemokine receptor. Using membranes from HEK-293 cells expressing CCR1, the ability of MPIF-1 to compete with [125I]MIP-1α binding was investigated (Fig. 2A). The competition curves observed with increasing concentrations of
MIP-1α or MPIF-1 were similar, in that 50% displacement of the bound [125I]MIP-1α was achieved with 50 nM MIP-1α or MPIF-1. At a concentration of 500 nM, MIP-1α or MPIF-1 displaced between 70–80% of the bound [125I]MIP-1α. In contrast, IL-8 in the same concentration range did not displace any [125I]MIP-1α (data not shown). Since the N-terminal region of β-chemokines is known to be important in receptor binding and activation, we compared the binding characteristics of MPIF-1 to those of the full-length protein (1–99) on CCR1-containing membranes. In competition binding experiments using [125I]MIP-1α and CCR1-containing membranes, the displacement curves generated with the two proteins were not significantly different (Table I). Using membranes from CCR2b-transfected HEK-293, 10 nM MCP-1 displaced 50% of the bound [125I]MCP-1, whereas MPIF-1 did not displace a significant amount of the bound iodinated ligand from CCR2b even at concentrations as high as 1000 nM (Fig. 2B). The binding selectivity of MPIF-1 for CCR5 was investigated by examining the ability of MPIF-1 to displace either [125I]MIP-1α or [125I]MIP-1β (Fig. 2C, right or left panel, respectively). Although the unlabeled chemokines effectively compete with their respective iodinated counterparts for binding to CCR5, MPIF-1, even at a 2000-fold excess, did not displace a significant amount of either bound [125I]MIP-1α or [125I]MIP-1β. Furthermore, the full-length form of the protein did not displace a significant amount of bound [125I]MIP-1α from CCR5 (Table I). These results demonstrate that the binding characteristics of MPIF-14–99 and MPIF-11–99 are the same on CCR1 and CCR5 and suggest that a shared MIP-1α/ MPIF-1 binding site on monocytes and dendritic cells is CCR1.

When membrane binding experiments were conducted at 24 or 37°C, the amount of [125I]MPIF-1 bound reached equilibrium within 30 min and remained stable for at least 90 min. Although the level of binding at 4°C was initially lower than that observed at 24 or 37°C, equivalent levels of [125I]MPIF-1 binding were observed at equilibrium (data not shown). Steady state binding experiments were conducted to determine the affinity of [125I]MPIF-1 for CCR1, and Fig. 3A depicts the binding isotherm. Conversion of the data by Scatchard analysis revealed a single affinity binding site with a Kd value of 745 pM (Fig. 3A, inset). The binding characteristics of iodinated MPIF-1 were further investigated in cross-competition studies using membranes from CCR1-transfected HEK-293 cells (Fig. 3B). In general, the competition curves for MPIF-1 and MIP-1α were similar; however, MPIF-1 was somewhat more effective in displacing the bound [125I]MIPF-1 than was MIP-1α. At a concentration of approximately 600 nM, MCP-3 displaced 50% of the bound [125I]MIPF-1. MIP-1β at the highest concentration tested only displaced 20% of the bound [125I]MIPF-1 from CCR1, consistent with its relatively weak affinity for CCR1.

Calcium flux in HEK-293 cells stability transfected with chemokine receptors

To test whether CCR1 is a functional receptor for MPIF-1, we measured calcium flux in CCR1-transfected HEK-293 cells after stimulation with MPIF-1. MPIF-1 induces a rapid calcium flux in

### Table I. Binding activities of MPIF-11–99 and MPIF-124–99 on CCR1 and CCR5

<table>
<thead>
<tr>
<th>MPIF-1 (nM)</th>
<th>MPIF-1α</th>
<th>MPIF-1β</th>
<th>MPIF-124–99</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>35/0</td>
<td>35/0</td>
<td>35/0</td>
</tr>
<tr>
<td>100</td>
<td>72/0</td>
<td>72/0</td>
<td>72/0</td>
</tr>
<tr>
<td>1000</td>
<td>90/0</td>
<td>90/0</td>
<td>90/0</td>
</tr>
</tbody>
</table>

*Binding experiments were conducted as outlined in Materials and Methods using 25 or 50 μg/determination of CCR1 or CCR5 containing membranes, respectively.*
these cells, but not in untransfected HEK-293 cells or in cells expressing CCR5 (Fig. 4A). Also, 4DE4 cells expressing the I-309 receptor (Fig. 4A) and HEK-293 cells expressing CX3CR1 (data not shown) failed to respond to MPIF-1. The maximal receptor stimulation obtained with 100 nM MPIF-1 was similar in magnitude to that obtained with the same concentration of the other CCR1 agonists, RANTES, MIP-1α, and MCP-3. Consistent with this, MPIF-1 pretreatment was able to desensitize CCR1 to subsequent stimulation with itself or RANTES, MIP-1α, or MCP-3, whereas it had no effect on the response of CCR5, CCR8, or CX3CR1 to known agonists. The calcium response elicited by MPIF-1 treatment in CCR1-expressing cells was dose dependent with a half-maximal effective concentration of approximately 25 nM (Fig. 4B).

Calcium flux in monocytes, dendritic cells, and THP-1 cells in response to MPIF-1

To identify any potential difference in the biological activities of MPIF-1_{24-99} and the full-length protein, we compared their abilities to induce cellular responses and their patterns of receptor desensitization on monocytes. The two forms of the protein had essentially identical potencies for inducing a Ca^{2+} flux and they demonstrated equivalent activity in the induction of F-actin polymerization (Table II; see below for details concerning F-actin experiments). Furthermore, they induced equivalent patterns of receptor desensitization.
(Table III). When the cells were treated first with MIP-1α or MCP-3, the calcium flux response to MPIF-1 was completely inhibited. In contrast, treating the cells first with MCP-1 induced a partial desensitization of the MPIF-1 response, while pretreatment with MIP-1β, even at a concentration as high as 100 nM, did not produce a significant reduction in the response to MPIF-1. Furthermore, the pattern of receptor desensitization induced by MPIF-1 was characterized in dendritic cells and THP-1 (Table IV). In general, the response pattern was equivalent to that observed in monocytes. However, cross-desensitization between MPIF-1 and MCP-1 in dendritic cells could not be studied, since MCP-1 does not induce calcium flux in dendritic cells, as previously reported (7). The same limitation was used when using MPIF-1β and THP-1 cells. The results from the Ca2+ flux and receptor desensitization studies are consistent with the data from the binding studies in demonstrating that both forms of MPIF-1 bind to a subpopulation of receptors that also bind MIP-1α.

**Evidence that MPIF-1 interacts with a G protein-coupled receptor**

The regulation of MPIF-1 binding to CCR1 by G proteins was tested by preincubating membranes from CCR1-transfected HEK-293 cells with GTP-γ-S and then with [125I]MPIF-1. GTP-γ-S inhibited [125I]MPIF-1 binding in a concentration-dependent manner with an IC50 of 10 nM (Fig. 5A). The involvement of G proteins in the biological response to MPIF-1 was further identified by testing the effect of pertussis toxin (PTX), which binds to and inactivates Gs and Gi proteins, on the MPIF-1-associated change in [Ca2+]. Preincubation of monocytes with PTX completely inhibited the calcium flux associated with MPIF-1 treatment (Fig. 5B).

**Table II. Comparison of the biological effects of the two forms of MPIF-1 on monocytes**

<table>
<thead>
<tr>
<th>Chemokine Treatment</th>
<th>Ca2+ Flux, F340/F380</th>
<th>Actin Polarization, Relative F-Actin Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPIF-1 1–99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.30</td>
<td>1.25</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>1.25</td>
<td>1.72</td>
</tr>
<tr>
<td>1000 ng/ml</td>
<td>1.50</td>
<td>2.36</td>
</tr>
<tr>
<td>MPIF-1 24–99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>0.40</td>
<td>1.30</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>1.25</td>
<td>1.84</td>
</tr>
<tr>
<td>1000 ng/ml</td>
<td>1.50</td>
<td>2.53</td>
</tr>
</tbody>
</table>

* Monocytes (1 x 10⁶/sample) were stimulated with the two recombinant MPIF-1 proteins and the response, based on Ca2+ flux and actin polymerization, was quantitated as described in Materials and Methods.

**Table III. Pattern of cross desensitization of the Ca2+ flux induced in monocytes by the two forms of MPIF-1**

<table>
<thead>
<tr>
<th>Chemokine Treatment</th>
<th>MPIF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>+ +</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>+ +</td>
</tr>
<tr>
<td>MCP-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MCP-3</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
</tbody>
</table>

* Fura-2 labeled monocytes were stimulated with the chemokines (100 nM) at 2-min intervals. Changes in Ca2+ flux are expressed as follows: +, response; +, half-maximal response; +, maximal response; ND, not determined.

**Table IV. Cross desensitization of Ca2+ flux induced by MPIF-1 in dendritic cells or THP-1 cells**

<table>
<thead>
<tr>
<th>Chemokine Treatment</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ND</td>
</tr>
<tr>
<td>MCP-3</td>
<td>++ + +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ + ND</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ + +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ + +</td>
</tr>
<tr>
<td>MIPF-1</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

* Cells labeled with Fura-2 were stimulated with chemokines at 2-min intervals. The chemokines were used at the following concentrations: MIPF-1 and MIPF-1α, 1000 ng/ml; MIPF-1α, MCP-1, and MCP-3, 100 ng/ml. The concentrations selected produced a comparable mobilization of intracellular Ca2+ in the target cells. Changes in Ca2+ flux are expressed as: +, maximal response; +, half-maximal response. ND, not determined.

**Downstream signaling events triggered by MPIF-1**

The increase in [Ca2+]i, that is associated with MPIF-1 receptor activation may be derived from intracellular and/or extracellular calcium pools. To determine whether the influx of extracellular calcium is the source of the [Ca2+]i, associated with the MPIF-1 response, monocytes were loaded with fura-2, rinsed with flux buffer without calcium and containing 2 mM EGTA, and subsequently stimulated with MPIF-1 (Fig. 6B). Under these conditions the [Ca2+]i response was less than the response seen in the presence of extracellular calcium and in the absence of EGTA (Fig. 6A). However, a residual peak of [Ca2+]i was reproducibly observed when monocytes were treated with MPIF-1, suggesting that a portion of the calcium response is mobilized from an intracellular source(s). The activation of PLC induces the formation of inositol triphosphate that subsequently stimulates the release of calcium from intracellular compartments. To ascertain whether MPIF-1 activates PLC, monocytes were pretreated with U73122, a PLC inhibitor, and then with MPIF-1 in the absence of extracellular calcium. Under these conditions, MPIF-1 did not induce an increase in [Ca2+]i (Fig. 6C). In contrast, when the cells were pretreated with U73343, a weakly acting analogue of U73122, and then with MPIF-1, there was no reduction in the intracellular derived [Ca2+]i, peak (Fig. 6D).

Since the stimulation of PLC is known to subsequently result in the activation of PKC, we investigated whether PKC activity modulated the calcium response of the target cells to MPIF-1 treatment. When monocytes were pretreated with the phorbol ester, PMA, the increase in [Ca2+]i, associated with MPIF-1 treatment was completely inhibited (Fig. 7B). However, this concentration of PMA had no effect on the basal levels of intracellular calcium. The inhibitory effect of PMA was dose dependent, with an IC50 value of approximately 5 nM (data not shown). To ascertain whether the inhibitory effect of PMA requires the activation of PKC, the cells were incubated first with staurosporine, a PKC inhibitor, and then treated with PMA. Pretreatment with staurosporine did not modulate the MPIF-1-induced [Ca2+]i response in monocytes (Fig. 7C). However, staurosporine did block the inhibitory effect of PMA on the MPIF-1-induced increase in [Ca2+]i (Fig. 7D).

**Effect of MPIF-1 on cAMP formation and [3H]AA release**

The structural homology of CCR1 to neuropeptide Y and angiotensin II receptors, both of which bind and activate adenylate cyclase, and the regulatory effects that MIP-1α has on adenylate cyclase in MO7e CFC cells suggest that ligands binding CCR1...
may modulate cAMP in target cells (24, 25). The possibility that MPIF-1 also affects adenylate cyclase activity was investigated by treating monocytes, dendritic cells, or THP-1 cells with MPIF-1 in a wide range of concentrations in the presence or the absence of the phosphodiesterase inhibitor, isobutylmethylxanthine, for time intervals ranging between 5–240 min. No significant change in the level of cAMP was noted under any of the conditions tested (data not shown).

To investigate whether MPIF-1 activates PLA2 in monocytes, the cells were labeled overnight with [3H]AA and subsequently treated with chemokines for different lengths of time. Treatment with MPIF-1 (1000 ng/ml) produced a rapid time-dependent increase in the amount of [3H]AA present in the medium (Fig. 8A). The response was dose dependent, with concentrations from 10–1000 ng/ml inducing a linear increase in the released [3H]AA, and saturation was achieved with 1000 ng/ml of MPIF-1 (Fig. 8B). Pretreatment of the cells with manoalide, an inhibitor of PLA2, blocked the release of [3H]AA following MPIF-1 treatment. Furthermore, the stimulated release of [3H]AA requires extracellular calcium, since the cells were unresponsive to MPIF-1 in calcium-free medium containing 5 mM EGTA.

**MPIF-1 induces actin polymerization in monocytes**

Chemokine-induced changes in cellular morphology, which ultimately result in a chemotactic response, are produced in part by a reorganization of the actin microfilament system. Quantitation of the effects of MPIF-1 on F-actin formation is based on changes in the amount of Oregon Green 488-labeled phalloidin bound to permeabilized monocytes. A rapid dose-dependent increase in F-actin content was observed when monocytes were treated with MPIF-1. The response reached saturation within 10 s and then declined in a linear manner approaching baseline levels after approximately 3 min (Fig. 9A). The polymerization of F-actin induced by MPIF-1 was inhibited by 4-BPB and NDGA, inhibitors of PLA2, and of 5-, 12-, and 15-lipoxygenase, respectively (Fig. 9B). Furthermore, pretreating the cells with U73122 resulted in a near complete inhibition of the response.

**Discussion**

In this report we describe the identification of the receptor selectivity of MPIF-1 and the signal transduction pathway activated following MPIF-1 binding. The results from Ca2+ flux, competition binding, and receptor desensitization experiments demonstrate that MPIF-1 is a specific ligand for CCR1. The inhibitory effects of GTP-γ-S and PTX show that G protein(s) is involved in MPIF-1 binding and receptor activation. In addition, we identified that the
source of the $[\text{Ca}^{2+}]_i$ response associated with MPIF-1 treatment is from both extracellular and PLC-inhibitor sensitive intracellular pools. We also show that MPIF-1 induces a rapid increase in the release of $[^{3}\text{H}]\text{AA}$, demonstrating that PLA$_2$ activity is stimulated following treatment with this CC chemokine. Activation of PLA$_2$ is also required for the rapid MPIF-1-dependent F-actin formation in monocytes.

The pattern of receptor binding with monocytes, dendritic cells, or HEK-293 cells, expressing a single chemokine receptor, demonstrates that MPIF-1 binds to a subpopulation of receptors used by MIP-1$\alpha$. Cross-competition studies using membranes from cells expressing CCR1, -2b, or -5, clearly show that MPIF-1 binds to CCR1 and that MPIF-1 and MIP-1$\alpha$ have nearly equivalent affinities for this receptor. However, the displacement curve for MPIF-1 and $[^{125}\text{I}]\text{MIP-1$\alpha$}$ compared with that for MIP-1$\alpha$ was shifted to the right by as much as 2 logs in whole cell binding assays using monocytes, dendritic cells, or THP-1 cells, suggesting that in these cell types MIP-1$\alpha$ has a greater affinity for CCR1 compared with MPIF-1. Since the affinity constants reported for $[^{125}\text{I}]\text{MIP-1$\alpha$}$ and CCR1 are in the range of those determined in this study for $[^{125}\text{I}]\text{MPIF-1}$ and CCR1, an explanation for the differences in the displacement curves based on a difference in affinities is not tenable (26). It is possible that monocytes and dendritic cells may have additional extracellular matrix components (e.g., glycosaminoglycans) that may affect MPIF-1 binding (27).

Alternatively, since the receptor selectivity of MIP-1$\alpha$ is broader than that exhibited by MPIF-1, the displacement curve with MIP-1$\alpha$ would be less pronounced in cells expressing multiply chemokine receptors.

The pattern of receptor selectivity of both forms of MPIF-1 characterized in binding assays is consistent with their receptor specificity as identified on the basis of $[\text{Ca}^{2+}]_i$ flux studies. In experiments with HEK-293 cells transfected with CCR1, MPIF-1 induces a $[\text{Ca}^{2+}]_i$ response, while cells transfected with CCR5 are unresponsive. Furthermore, MPIF-1, MIP-1$\alpha$, RANTES, and MCP-3 symmetrically cross-desensitize each other on CCR1. The calcium desensitization reported in this study is different from that previously reported by Forssmann et al. (17). In the latter study, prestimulation of monocytes with MPIF-1 only partially inhibits the response to a subsequent challenge with MIP-1$\alpha$ and completely desensitizes the cells toward MIP-1$\beta$. On the basis of our experiments involving calcium flux with HEK-293 cells expressing CCR5, no interaction was detected between MPIF-1 and CCR5. Similarly, neither full-length MPIF-11–99 nor MPIF-124–99 demonstrated any affinity for CCR5 in binding assays. In contrast, in displacement studies using CCR1-containing membranes,
MPIF-1 or the full-length protein produced an equivalent displacement of [125I]MIP-1α. Thus, the change in the N-terminal sequence of MPIF-1 does not produce a change in its receptor specificity or in its apparent affinity for CCR1.

The remodeling of membrane phospholipids by PLA2, PLC, and PLD is an essential step in the signal transduction pathways associated with the response of monocytes to CC chemokines and leads to the induction of diverse biological responses, including chemotaxis, activation of oxidative burst, and release of lysosomal enzymes (6). We have shown that both PLC and PLA2 are involved in the response of monocytes to MPIF-1. Approximately 30% of the [Ca2+]i response to MPIF-1 is derived from a PLC-sensitive intracellular pool. It is not known whether an increase in this intracellular pool of calcium has a preferential biological effect compared with the calcium derived from extracellular sources. How- ever, pretreating the cells with the PLC inhibitor, U73122, blocks the MPIF-1-induced chemotaxis of dendritic cells (data not shown). Once formed, AA is further processed by both cyclo-oxygenases and lipoxygenases. The F-actin remodeling response observed following epidermal growth factor stimulation of A431 cells and neutrophils involves both 5-lipoxygenase and cyclo-oxygenase metabolites of AA (33, 34). The inhibition of 5-, 12-, and 15-lipoxygenases with NDGA results in the complete blockage of F-actin formation. The findings that some of the lipoxygenase metabolites of AA (e.g., 5-oxo-eicosatetraenoic acid and 5-hydroxyeicosatetraenoic acid) induce monocyte chemotaxis and AA release and act synergistically with CC chemokines indicate that they serve not only to amplify the response to CC chemokines in target cells but also as intracellular messengers affecting other cell types that potentially are unresponsive to the original chemokine (35).

The CCR1 expression pattern, based on immunofluorescence or RT-PCR, in peripheral blood is restricted to monocytes, T lymphocytes, and NK cells and in bone marrow and cord blood to CD34+ cells, erythroblast, and erythroid progenitor cells (36, 37). On the basis of the binding data and second messenger studies presented here for MPIF-1 demonstrating a selectivity for CCR1, other cells expressing CCR1 are likely to be targets for this chemokine, and further comparison of the biological activity of MPIF-1 to MIP-1α would be of interest.

Acknowledgments

We thank Katherine McKinnon and Jeffrey Carrell for the FACS analysis. We also thank Drs. Deborah Russell and Gianni Garotta for their critical evaluation of the manuscript.

References
